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Potential cosmeceutical applications of a water extract of *Duchesnea indica* cultivated in Jeju Island

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ABSTRACT

The purpose of this study was to investigate the anti-aging, anti-bacterial, and anti-inflammatory effects of a hot water extract of Duchesnea indica (DIW). The anti-aging properties of this extract were assessed as anti-elastase, 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging, 2,2-azinobis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS) scavenging, and pigmentation activities. We also investigated the anti-inflammatory potential of DIW in lipopolysaccharide-stimulated RAW 264.7 macrophage cells. DIW possessed high elastase inhibitory, and DPPH and ABTS radical scavenging activities (IC_{50}s; 35.1, 19.2, and 84.0 µg/mL, respectively). We determined the anti-bacterial activities of DIW using minimum inhibitory concentration (MIC) values. DIW showed excellent antibacterial activities against drug-susceptible and -resistant skin pathogens such as Propioni bacterium acnes, Staphylococcus epidermidis, and Staphylococcus aureus, with MICs ranging from 78.1 to 1250 µg/mL. DIW significantly stimulated melanin synthesis of B16 mouse melanoma cells, and inhibited nitric oxide synthesis of RAW 264.7 macrophage cells, in a concentration-dependent manner. DIW also effectively attenuated expression of the inflammation-mediating enzyme, inducible nitric oxide synthase, at the protein level in a concentration-dependent manner. The high-performance liquid chromatography fingerprint of DIW indicated the presence of quercetin and kaempferol at 25.3 and 12.0 µg/mL, respectively. To evaluate the safety of DIW, the human skin primary irritation test was performed on normal skin (upper back) of 31 volunteers to determine if any constituent presented an irritation or sensitization potential. DIW did not induce any adverse reactions at 100 µg/mL. Taken together, our results suggest that DIW may be considered as an anti-aging and anti-inflammatory candidate for cosmeceutical applications.

Keywords: Anti-aging, *Duchesnea indica*, HPLC fingerprint, human skin primary irritation tests, inflammation, minimum inhibitory concentration

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INTRODUCTION

Skin is an important barrier that protects the body from damage due to direct contact with the outside environment including a broad spectrum of physical (ultraviolet (UV) radiation) and chemical (xenobiotic) agents that are capable of altering its structure and function [1]. Many environmental pollutants catalyze the production of reactive oxygen species (ROS) directly or indirectly, acting as cell-signaling molecules. These ROS ultimately react with DNA, proteins, and lipids causing oxidative damage, disrupting regulatory pathways, and altering the differentiation, proliferation, and apoptosis of skin cells [2-3]. Whereas, the skin possesses a number of defense mechanisms that interact with environmental pollutants to neutralize their deleterious effects. These defense mechanism include non-enzymatic and enzymatic molecules that function as potent antioxidant or oxidant-degrading systems [1,4]. For screening antioxidants, the methods based on a single relatively stable reagent such as DPPH• and ABTS•+ have most popular, because of their simple set-up and ease of control [5-6]. For example, degradation of elastin, due to elastase secretion and activation upon exposure to UV light or ROS, is a major cause of loss of skin tone and

elasticity, resulting in visible signs such as wrinkling and sagging [7]. Thus, an approach that inhibits elastase activity could be applied as a useful strategy to protect against skin aging[6,8].

Another important issue besides skin aging is skin pigmentation, which impacts the health and well-being of an individual significantly. Pigments, such as melanin, synthesized by cutaneous melanocytes protect the individual from various environmental pollutants that can damage cells and cause cancer and skin aging. Melanin, an effective scavenger of free radicals, effectively absorbs UV light penetrating the skin and prevents consequent DNA damage. A deficiency of epidermal melanin increases susceptibility to skin cancers, and is an indicator of skin aging [9-10].

Acne vulgaris, a common inflammatory skin disease, is also a very concerning condition and is attributed to multiple factors, such as inflammatory processes and the proliferation of skin pathogens within follicles. The widespread use of antibiotics in acne vulgaris has resulted in the emergence of multi-drug-resistant pathogens that can negatively impact human skin health through an increased incidence of treatment failure and more severe disease [11-12]. Therefore, many researchers are increasingly turning their attention to alternative medicines to develop better treatments against drug-resistant pathogens and inflammatory events.

Recently, medicinal plants and their natural constituents have received positive attention for several cosmetic applications; e.g., hair depigmentation through tyrosinase inactivation and the subsequent inhibition of melanin synthesis, anti-aging by elastase inhibition, skin rejuvenation by antioxidant mechanisms, and the inhibition of skin pathogen growth and chronic inflammation thereby diminishing skin disease [13]. There exist several reports on the ability of medicinal plants and their active ingredients, either singly or in combination, to serve as cosmetic ingredients. Examples include quercetin, naringenin, rosmarinic acid, and nobiletin for hair depigmentation [9, 14-16], cocoa pod, *Astilbechinensis*, and *Areca catechu* extracts for anti-aging [17-19], *Prunuspadus* extract and curcumin for protecting against free radical damage [20-21], and *Neolitseasericea*, *Cryptomeria japonica*, and *Abieskoreana* essential oils for anti-acne activity [22-24]. Based on our interest in characterizing the bioactive secondary metabolites from Jeju medicinal plants for personal care uses, we have undertaken biological studies of the water extract of *Duchesnea indica* for cosmetic applications.

MATERIALS AND METHODS

Material preparation

D.indica plants were collected in July 2015 by the Tamnamo Company (Jeju-do, Korea) from Jeju Island. A voucher specimen (CSC-2015-006) has been deposited at the Cosmetic Sciences Center, Department of Chemistry and Cosmetics, Jeju National University. Prior to extraction, *D. Indica* plants were thoroughly washed, dried at room temperature for 2 weeks, and pulverized. The dried *D. Indica* (400 g) was extracted with distilled water at 70˚C for 4 h and then dried using a rotary evaporator. This extract, named DIW, yielded 92.4g (23.1% by weight).

Radical scavenging assays

The DPPH radical scavenging activity of DIW was determined according to the method of Ikram et al. [25],using minor modifications. Briefly, 180 µL of DPPH (0.2 mM in ethanol) was added to 20 µL of DIW solutions(10 to 1000 µg/mL) and reacted in the dark at 25°C for 10 min. The absorbance of the sample (Abs_{sample}), and a negative control ($Abs_{control}$) consisting of only distilled water, was measured at 517 nm using a microplate reader. The absorbance values were converted into percentage radical scavenging activity by using the following equation:

$$\text{DPPH scavenging activity} = [(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100$$

The radical scavenging activity of DIW was also determined using ABTS according to the method of Ikram et al.[25],using minor modifications. Briefly, 7.0 mM ABTS (in 2.45 mM potassium persulfate) was activated in the dark at 25°C for 16 h. The ABTS solution was diluted with ethanol to achieve a working solution with an absorbance at 700 nm of 0.78 ± 0.02. Then, 20µL of DIW solutions (10 to 1,000 µg/mL) were added to 180 µL of the working solution, reacted in the dark at 25°C for 15 min, and the absorbance of the sample and control (ethanol only) were measured. The absorbance values were converted into percentage radical scavenging activity by using the following equation:

$$\text{ABTS radical scavenging activity (\%)} = [(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100.$$

Tyrosinase assay

The activation of mushroom tyrosinase by DIW was determined using a method presented in the literature previously as an inhibition assay, with some modifications [26]. Briefly, mushroom tyrosinase (2500 units/mL, 5 µL) and L-tyrosine (70µL) were added to 105 µL phosphate buffer (0.1 M, pH 6.8) containing DIW (20 µL). The

test mixture (200 µL) was reacted for 10 ~ 15 min at 37°C,and absorbance from the formation of dopa-chrome was measured at 480 nm with a microplate reader. Similar mixtures without DIW, and a solution of arbutin (hydroquinone-O-β-glucopyranoside), were used as negative and positive controls, respectively. Each treatment was carried out three times. The percentage activation of tyrosinase was calculated using the following equation:

$$\text{activation}(\%) = [1 - (Abs_{sample} - Abs_{blank})/Abs_{control}] \times 100$$ where, Abs_{blank} is the absorbance of the blank.

Elastase inhibition assay

The inhibition of porcine pancreatic elastase activity by DIW was determined using an enzymatic colorimetric method with N-succ-$(ala)_3$-p-nitroanilide as the substrate. Briefly, porcine pancreatic elastase (0.1 mg/mL, 13 µL) and N-succ-$(ala)_3$-p-nitroanilide (6.25 mM, 10 µmL) were mixed with 157 µL Tris-hydrochloride buffer (0.2 M, pH 8) containing DIW (20 µL). The test mixture (200 µL) was reacted for 15 min at 25°C and absorbance from the formation of ρ-nitroaniline was measured at 405 nm with a microplate reader. A similar mixture without DIW was used as the control. Each treatment was carried out three times, and the percentage inhibition of elastase activity was calculated using the following equation:

$$\text{inhibition}(\%) = [1 - (Abs_{sample} - Abs_{blank})/Abs_{control}] \times 100.$$

Microorganisms and minimum inhibitory concentration determinations

Microorganisms were obtained from the Culture Collection of Antimicrobial Resistant Microbes (CCARM, Seoul, Korea). *Propioni bacterium acnes* [CCARM 0081, CCARM 9009, CCARM 9010, and CCARM 9089], *Staphylococcus epidermidis* [CCARM 3709, CCARM 3710, and CCARM 3711], and *Staphylococcus aureus*[CCARM 3032, CCARM 3926, and CCARM 3842], that are well-known to cause acne, were chosen as test microorganisms according to their pathological capacity. *Propioni bacterium* strains were incubated at 37°C for 48 h in Gifu anaerobic agar medium (Nissui Pharmaceutical, Tokyo, Japan) under anaerobic conditions. Other bacteria were incubated at 37°C for 24 h in Tryptic soy agar medium (Difco, Bordeaux, France) under aerobic conditions before implementing the assay. The antimicrobial activities of DIW against skin pathogens (*P. acnes, S. epidermidis,* and *S. aureus*) were determined via the broth dilution method. The minimum inhibitory concentration (MIC) was determined as the lowest concentration of test samples resulting in complete inhibition of bacterial growth by the optical density that was measured spectrophotometrically at 600 nm after 48 (*P. acnes)* or24h (*S. epidermidis* and *S. aureus*) of incubation.

Melanin content assay

The murine B16F10 melanoma cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were grown at 37°C in a humidified atmosphere containing 5% CO_2 in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (GIBCO, Grand Island, NY, USA), 2 mM glutamine, 1% non-essential amino acids (100×), and 1% sodium pyruvate (complete RPMI).B16F10 cells (2.0×10^4/mL) were seeded into a plate with medium, and stimulated with α-melanocyte-stimulating hormone (500 nM). Cells were incubated with DIW (50, 100, and 200 µg/mL) at 37°C for 72 h, then washed in ice-cold phosphate-buffered saline. Washed cells were incubated at 80°Cfor 1 h in 1 mL of 1 N NaOH/10% dimethyl sulfoxide and then vortexed to solubilize the melanin. The absorbance of the resulting solution was measured at 450 nm. Melanin content was determined based on absorbance/µg of protein in the extract from each treatment. The protein concentration of the cells was determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as the standard.

Nitric oxide production

RAW264.7 cells, a murine macrophage-like cell line, were obtained from the Korean Cell Line Bank (Seoul, Korea). Cells were maintained in a Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (GIBCO) and 1% penicillin at 37°C in a humidified 5% CO_2 incubator. The cells were subcultured every 3days. Nitric oxide (NO) production in the RAW264.7 cell culture supernatant was determined as described previously [22-24] using the Griess reaction [27]. Briefly, RAW 264.7 cells (1.5×10^5/well) were plated in 24-well plates and incubated with different concentrations of DIW (12.5, 25, 50, 100, 200, and 400 µg/mL) and 1 µg/mL of LPS for 24 h. Cell culture medium (100 µL) was mixed with 100 µL of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediaminedihydrochloride in 2.5% phosphoric acid). The mixture was then incubated at room temperature for 10 min and the absorbance at 540 nm was measured in a microplate reader. Fresh culture medium was employed as a blank in every experiment. The quantity of nitrite was determined from a sodium nitrite standard curve.

Cell viability

Cytotoxicity induced by DIW was analyzed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zoliumbromide

(MTT) assay. This assay is based on the reduction of MTT to formazan by mitochondrial dehydrogenases. RAW 264.7 cells (1.5 $\times 10^5$/well) were plated into 96-well plates containing Dulbecco's modified Eagle's medium and incubated overnight. Cells were then treated with LPS and various concentrations of DIW, followed by incubation for 24 h at 37°C. The MTT reagent (0.5 mg/mL) (Sigma-Aldrich, St. Louis, MO, USA) was added to the medium and incubated for an additional 4 h. Cell-free supernatants were then removed and cells extracted with DMSO. The extent of MTT reduction to formazan was determined by measuring the absorbance at 540 nm using a microplate reader.

Western blot analyses

RAW 264.7 cells (1.0×10^6/well) were preincubated in a 60-mm petri dish in an atmosphere of 5% CO_2 at 37°C for 18 h. After removal of the culture medium, the cells were incubated in medium with different concentrations of DIW (25, 50, 100, and 200 μg/mL) and 1 μg/mL of LPS for 24 h. The cells were then collected and washed twice with cold phosphate buffered saline. Cell lysates were centrifuged and protein concentrations determined using a Bio-Rad protein assay kitwith bovine serum albumin as the standard. Protein (30–40 μg) was applied to a 10% sodium dodecyl sulfate-polyacrylamide gel and transferred onto a polyvinylidene fluoride membrane for 2 h. The membranes were blocked with 5% skim milk in Tris-buffered saline containing Tween-20 at 4°C overnight and then incubated with an anti-mouse iNOS antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h at room temperature. The membranes were then washed three times and incubated with a horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (1:5000; Vector Laboratories, Burlingame, CA, USA) for 1 h. Protein bands were visualized using an enhanced chemiluminescence western blotting detection kit (Amersham Biosciences, Piscataway, NJ, USA).

High-performance liquid chromatograph fingerprinting

High-performance liquid chromatography (HPLC) was performed with a Waters 2695 chromatograph system (Waters, Milford, MA, USA) including a photodiode array detector, an Empower 2 Pro System, and an In-Line Degasser 4 chamber. A reversed-phase Kromasil 100-5-C_{18} column (250 × 4.6 mm) was used to separate compounds from DIW. Quantitative analysis used a binary solvent system of (A) water containing 0.1% acetic acid, and(B) methanol with the following gradient elution: 0–5 min, 60% A, 40% B; 5–35 min, 60–30% A, 40–70% B; 35–40 min, 0% A, 100% B. The injection volume of the sample was 10.0 μL. The column and oven temperatures were controlled at 25 and 40°C, respectively. The flow rate was kept at 1.0 mL/min and detection of the two compounds (quercetin and kaempferol) was carried out at 260 nm. All solutions, including samples and standards, were filtered through a 0.45 μm membrane before direct injection into the HPLC system.

Human skin primary irritation test

The human skin irritation test for DIW was performed by Dermapro (Seoul, Korea) based on the Declaration of Helsinki principles. Thirty-one healthy female Korean subjects were selected based on inclusion and exclusion criteria and written consent was obtained in each case. The average age was 43.8 ± 4.3 years (range: 36–50 years). DIW formulated with 50% butylene glycol was prepared and 30 mL at concentrations of 100 and 200 μg/mL were applied to each subject. The patches (chambers) remained in place for 48 h. Irritation readings were scored according to the criteria of Frosch and Kligman[28], and Personal Care Product Council (PCPC) guidelines.

Table 1. Antimicrobial activity of *Duchesnea indica* water extract (DIW)

Strains		Drug-resistance patterns of skin pathogens (MIC; μg/mL)	MIC values of DIW (μg/mL)
Staphylococcus epidermidis	CCARM 3709	susceptible	1250
	CCARM 3710	erythromycin (>32), clindamycin (>16), chloramphenicol (64)	312.5
	CCARM 3711	tetracycline (>32)	N.D.
Propionibacterium acnes	CCARM 0081	susceptible	N.D.
	CCARM 9009	clindamycin (≥64)	156.3
	CCARM 9010	clindamycin (≥64)	N.D.
	CCARM 9089	clindamycin(128), erythromycin(≥128)	N.D
Staphylococcus aureus	CCARM 3032	erythromycin(≥128)	78.1
	CCARM 3926	chloramphenicol(2.0), penicillin(2.0)	78.1
	CCARM 3842	chloramphenicol(4.0), penicillin(8.0)	78.1

Statistical analysis

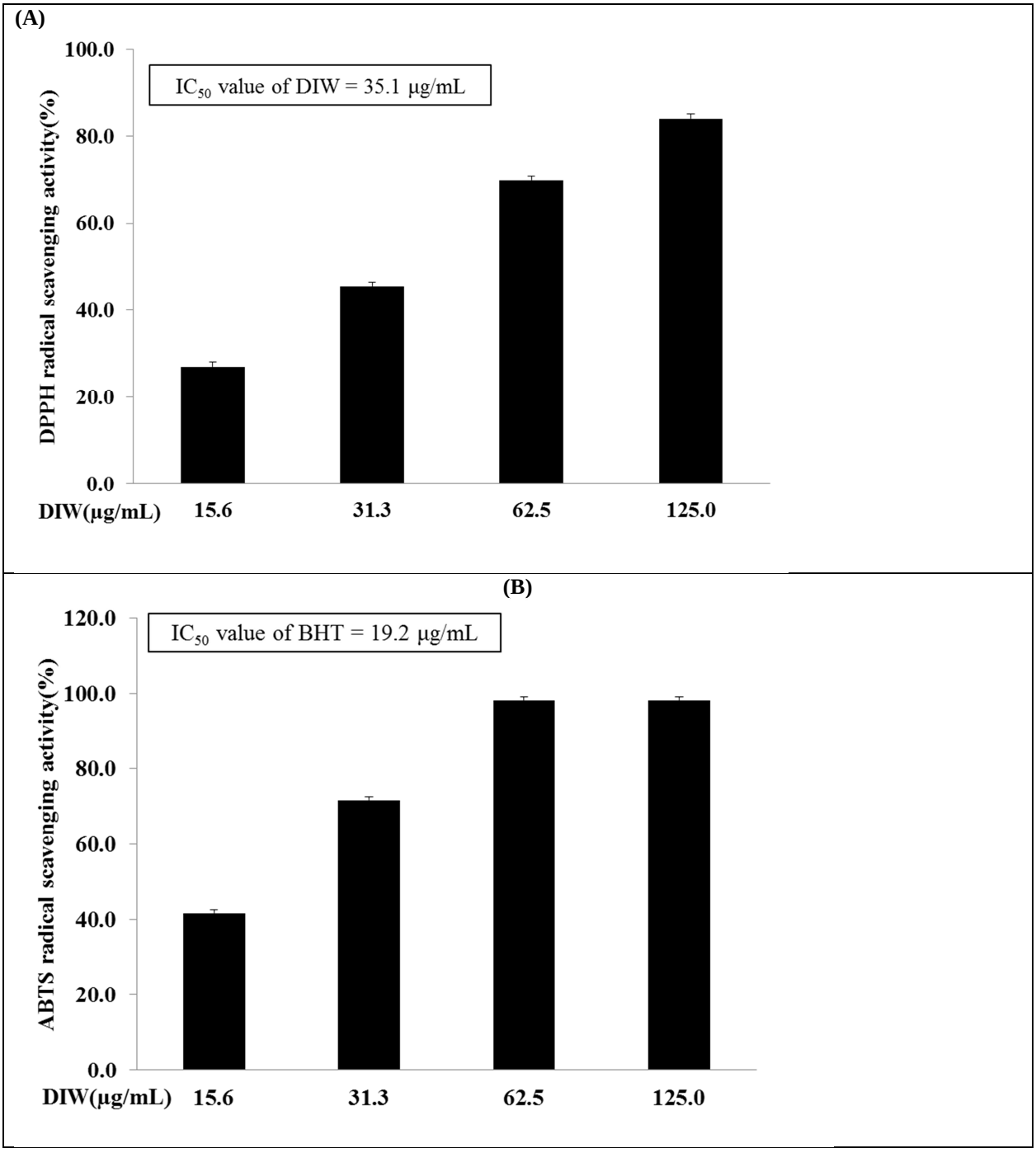
All tests were carried out in triplicate and results are presented as means ± standard error of the mean (SEM) of 3 independent experiments. Statistical comparisons between groups were performed using Student's t test. P less than 0.05 was considered significant.

Table 2. Results of the human skin primary irritation test (*n* = 31)

Test Material	No. of responders	48 h				72 h				Reaction grade[a]		
		1+	2+	3+	4+	1+	2+	3+	4+	48 h	72 h	Mean
50% butylene glycol (vehicle control)	2	1[b]	-	-	-	2	-	-	-	0.8	1.6	1.2
Negative control	0	-	-	-	-	-	-	-	-	0	0	0
DIW (100 μg/mL)	0	-	-	-	-	-	-	-	-	0	0	0
DIW (200 μg/mL)	2	-	-	-	-	2	-	-	-	0	1.6	0.8

[a]*Reaction grade = Σ[{Grade × no. of responders}/{4 (maximum grade) ×31 (total subjects)}] ×100 ×(1/2).*
[b]*No reaction.*

Fig 1.Antioxidant and elastaseinhibitory activities of *Duchesnea indica* water (DIW) extracts. The antioxidant capacity of DIW was measured using the (A) DPPH and (B) ABTS scavenging assays. (C) Inhibition of porcine pancreatic elastase by DIW



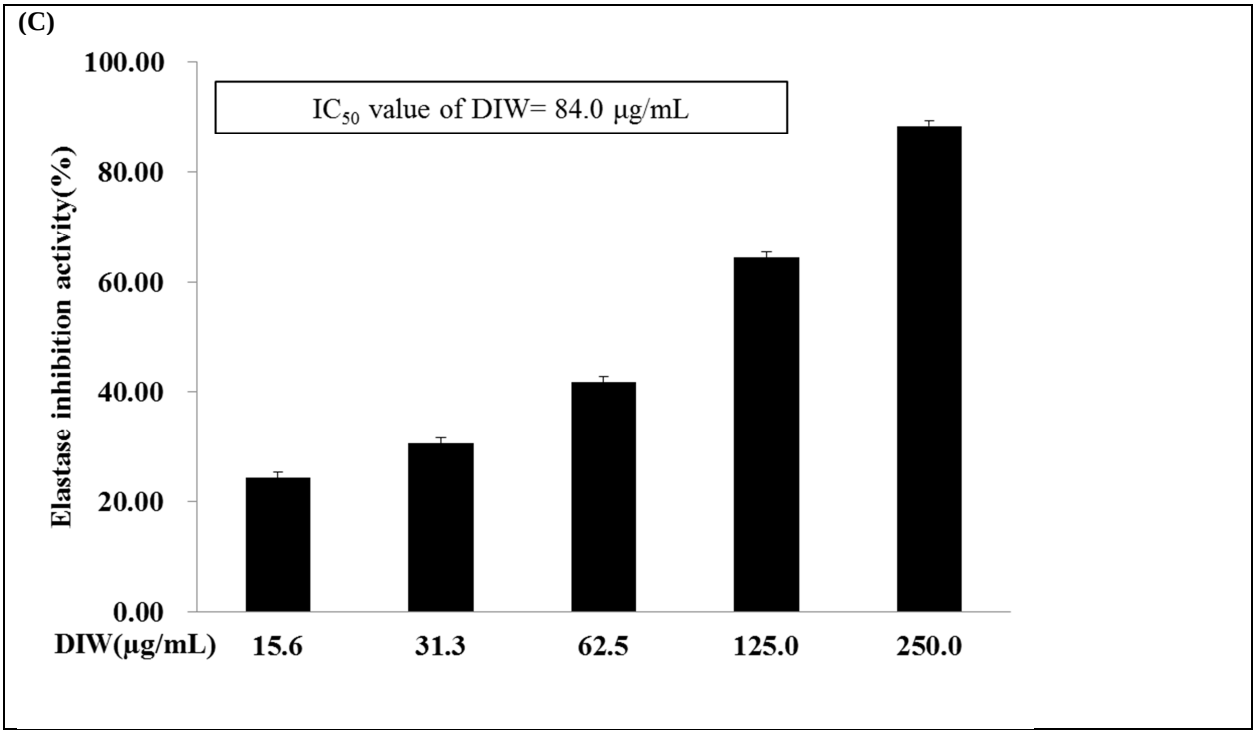
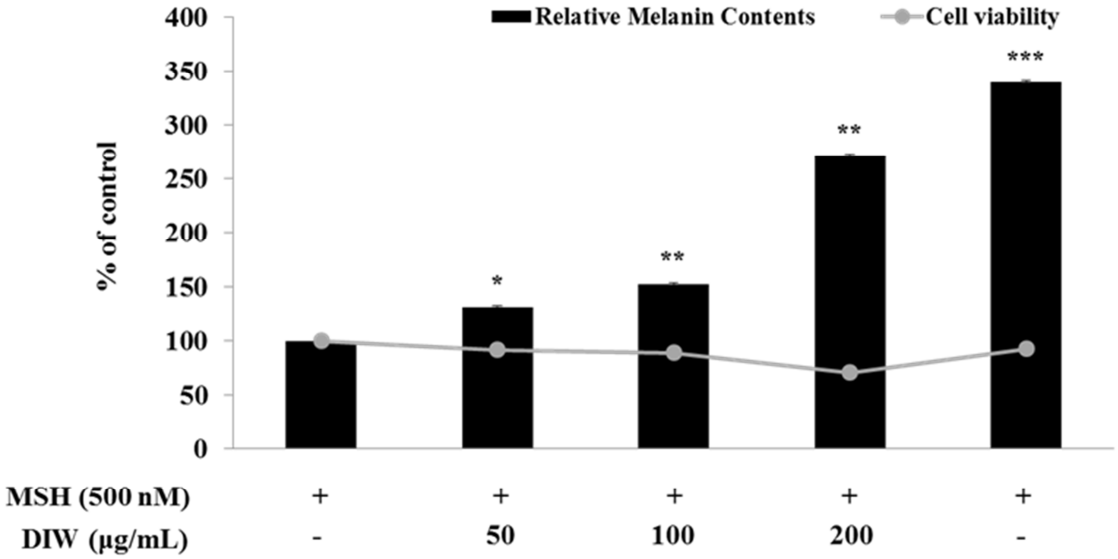


Fig 2. Effects of *Duchesnea indica* water (DIW) extracts on melanogenesis of B16F10 cells and on mushroom tyrosinase activity. (A) B16F10 cells (2.0×10^4/mL) were pre-incubated for 18 h. The melanin content in these cells was assayed after further incubation with α-melanocyte stimulating hormone (500 nM) and DIW for 72 h at 37˚C in a 5% CO_2 atmosphere. Values are the means ± SEM of three independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001 versus control.(B) The potential effects of DIW on skin melanin production was determined based on its effects on mushroom tyrosinase activity. DIW increased tyrosinase activity dose-dependently up to 125 μg/mL. No further increases in tyrosinase activity were seen up to 1000 μg/mL.

(A)



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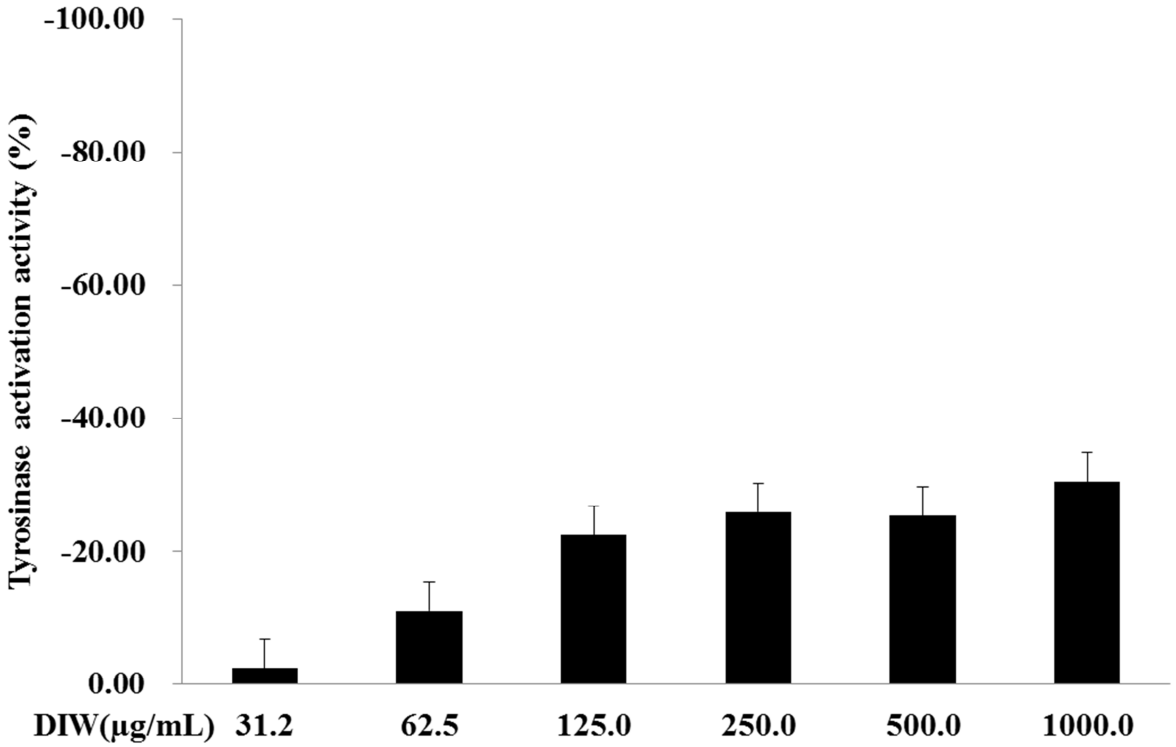
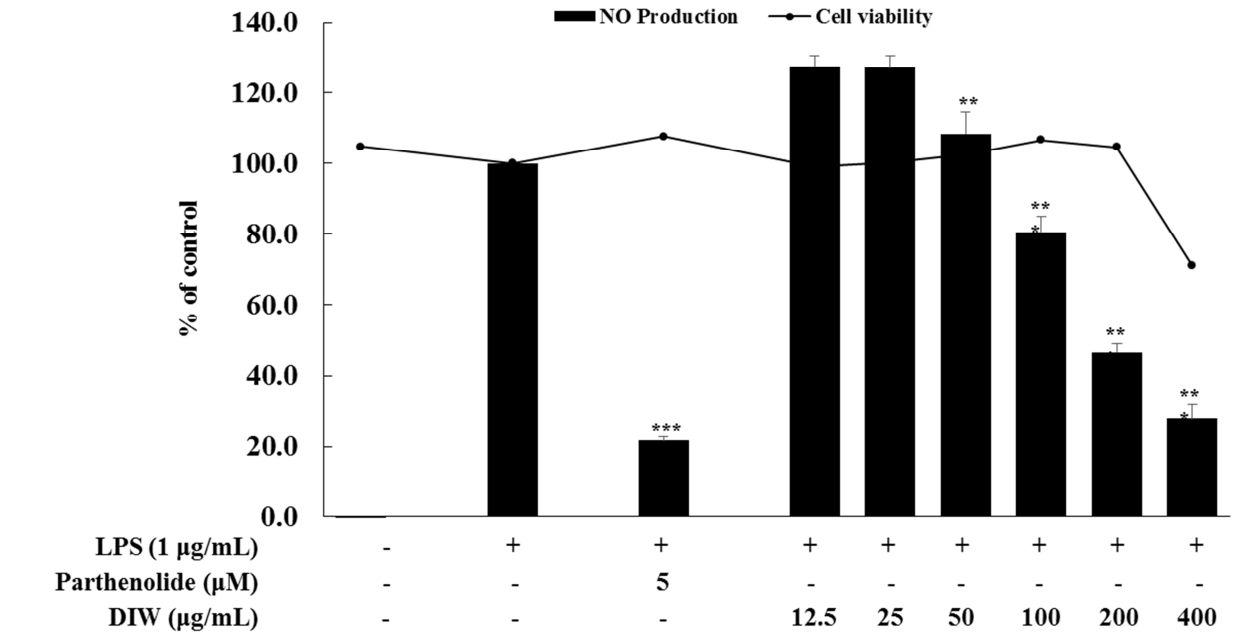


Fig 3. Effects of *Duchesnea indica* water (DIW) extracts on nitric oxide production, cell viability, and iNOS expression of lipopolysaccharide-stimulated RAW264.7 murine macrophages. (A) Cells (1.5 x 10^5/mL) were pre-incubated for 18 h. Nitric oxide production and cell viability were determined in cells stimulated with lipopolysaccharide (1 μg/mL) in the presence or absence of different concentrations of DIW for 24 h. Values are the means ± SEM of three independent experiments.*P < 0.05; **P < 0.01; ***P < 0.001 compared to LPS alone. (B) iNOS protein levels were analyzed by western blotting.

(A)



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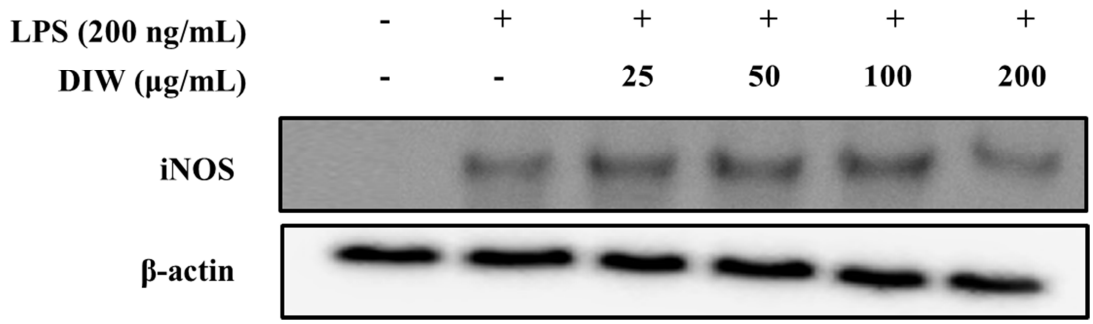
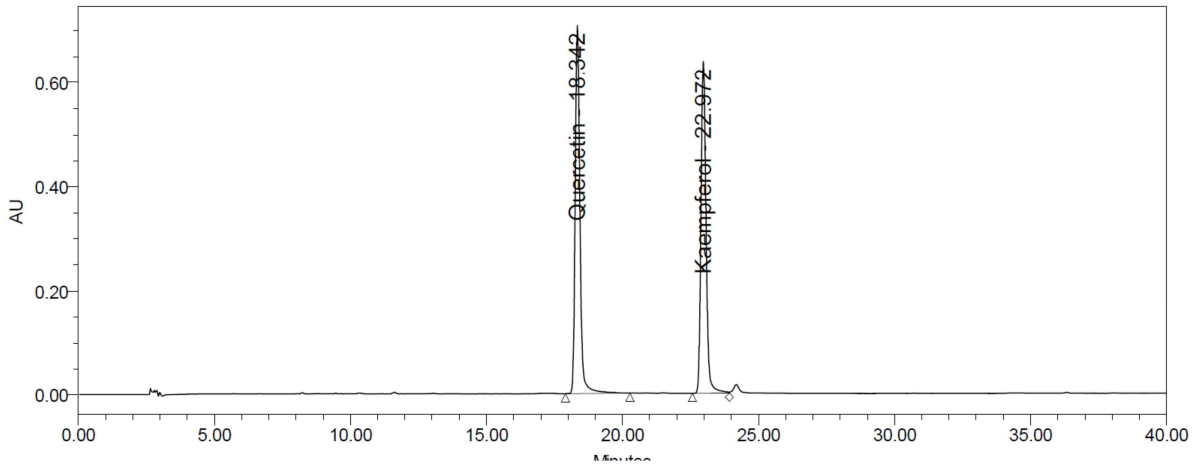
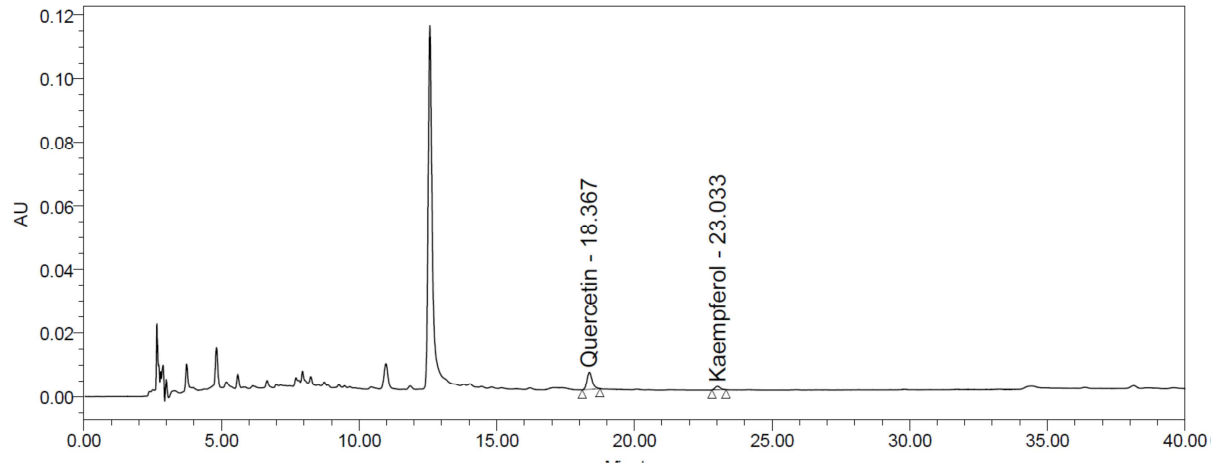


Fig. 4. HPLC fingerprinting analysis of *Duchesnea indica* water (DIW) extracts. (A)Quercetin and kaempferol standards. (B)Quercetin and kaempferol were detected in DIW. Detection was done at a wavelength of 260 nm

(A)



(B)



RESULTS AND DISCUSSION

Many studies have shown that natural antioxidants are closely related with anti-aging effects. Thus, antioxidant capacity is widely used as a parameter to characterize medicinal plants and natural products. In this study, DIW extracts were investigated to determine the anti-aging potential of this plant regarding its DPPH and ABTS scavenging activities. DPPH is a stable free radical and a change in its color in the presence of antioxidants is the most popular and authentic method of assessing radical scavenging [29]. The DPPH radical possess an unpaired electron that is responsible for its visible deep purple color. When DPPH accepts an electron donated by antioxidant materials, it is decolorized and this change can be measured quantitatively by differences in the absorbance at 540 nm [30]. In this assay, butylated hydroxytoluene is used as a standard antioxidant.

DIW scavenged DPPH radicals in a concentration-dependent manner (Fig. 1A). Scavenging activity was also expressed as a median inhibitory concentration (IC_{50}) value. These results showed that DIW exhibited high DPPH radical scavenging activity with an $IC_{50}$of 35.1 µg/mL. This was comparable to BHT(22.2µg/mL). The ABTS assay is also a widely used spectrophotometric assay for determining antioxidant activity. In this assay, the blue/green $ABTS^{+}$ radical, generated by oxidation of ABTS with potassium persulfate, is reduced in the presence of antioxidants, and the extent of decolorization is determined at a fixed time point [31]. The scavenging activities of DIW obtained using the ABTS method ranged from 41.6(15.6 µg/mL) to 98.2% (62.5 µg/mL)with an $IC_{50}$of 19.2 µg/mL (Fig. 1B). This was again comparable to theIC_{50} of BHT(23.0µg/mL).

Elastin is a protein found in connective tissue that is responsible for elasticity of the skin and lungs. This protein is degraded by the enzyme elastase, and this degradation increases with age and/or repeated UV-radiation exposures, leading to skin aging [32]. Therefore, the elastase inhibitory activity of DIW was studied in a cell free system. The inhibitory effects of DIW on porcine pancreatic elastase were increased with concentrations up to 250 µg/mL, with an $IC_{50}$of 84 µg/mL (Fig. 1C).

Tanning agents and treatments for hair depigmentation remain cosmetically desirable in many parts of the world and, more importantly, may improve the healthy and youthful condition of the human body. Melanin plays a role in protecting human skin from UV radiation. Its synthesis involves several enzymes such as tyrosinase, tyrosinase-related protein-1 (TRP-1), and TRP-2. Tyrosinase is the most important enzyme because melanin production is dependent on its expression and activation [33]. Therefore, this study was conducted to determine the effect of DIW on melanogenesis in B16 cells. As shown in Figure2, DIW has the ability to stimulate melanogenesis in B16 cells in a dose-dependent manner. At concentrations of 50, 100, and 200 µg/mL, the melanin content was increased by 32, 53, and 172%, respectively. In addition, the cell viability assay showed that, up to 200 µg/mL, the DIW extract was non-cytotoxic to B16 cells even after 48 h of incubation (Fig. 2A). We also observed that the increase in the melanin content was consistent with an increase in mushroom tyrosinase activity in the presence of DIW (Fig. 2B). These results showed that DIW has the potential to be used as a tanning agent or as a treatment for hair depigmentation.

Many people have acne vulgaris (or simply acne) at some point in their life. It is a long-term skin disease that can vary from mild to severe, and can affect the skin on the face, back, shoulders, and chest. Generally, an acne pimpled face is the result of the combined action of skin pathogens and the inflammatory response. Therefore, we examined whether DIW possessed anti-inflammatory and anti-microbial activities against skin pathogens. The in vitro antimicrobial activity of DIW against *P. acnes*, *S. aureus*, and *S. epidermidis* was assessed by the presence or absence of inhibition zones on cultured plates, and determination of the MIC values. The MIC was recorded as the lowest concentration of DIW that inhibited visible growth. As shown in Table 1, DIW exhibited moderate antibacterial activity against drug-susceptible and -resistant *P. acnes*, *S. aureus*, and *S. epidermidis*.

We next examined whether DIW had anti-inflammatory activity. To investigate the effect of DIW on NO production, we measured the accumulation of nitrite, a stable oxidized product of NO, in mouse macrophage RAW 264.7 cells stimulated with LPS for 24 h in the presence or absence of DIW. Nitrite levels in LPS-stimulated cells increased significantly compared to control cells (Fig. 3A). DIW (50, 100, 200, and 400 µg/mL) markedly inhibited LPS-induced NO production in a dose-dependent manner. The numbers of viable activated macrophages were not altered by DIW at any concentration, as determined by the MTT assay. This indicated that the inhibition of NO synthesis by DIW was not simply due to cytotoxic effects.

DIW effectively attenuated the expression of the inflammation-mediating enzyme, inducible nitric oxide synthase (iNOS), at the protein level, in a concentration-dependent manner (Fig 3B). Therefore, we suggest that DIW may be an attractive ingredient for diminishing skin inflammation such as occurs with acne vulgaris and atopic dermatitis.

Chromatographic methods (for example, HPLC fingerprinting) can be used to identify the active ingredients of traditional oriental medicines. In the natural chemistry area, interest in HPLC fingerprint analysis has increased, not only in Asia, but also around the world. Therefore, a simple HPLC fingerprint was developed. Because quercetin and kaempferol have been reported as effective anti-inflammatory ingredients in the Duchesnea plant, they were used as standard substances. Using HPLC fingerprinting, quercetin and kaempferol were resolved from the DIW extract with excellent peak shapes. The quercetin and kaempferol contents in the DIW extract were25.3 and 12.0 µg/mL, respectively (Fig. 4).

Finally, to assess the application of DIW as a cosmetic ingredient, we performed the primary skin irritation test on human skin. Human patch tests revealed that the application of DIW (1%) did not induce any severe adverse reactions (Table 2).

CONCLUSION

This is the first study investigating the antioxidant, anti-elastase, anti-acne, and hyper pigmenting activity of DIW. The free radical scavenging and elastase inhibitory activities of the *D. indica* extract suggest that they can help restore skin elasticity and thereby slow the wrinkling process. DIW also promoted melanin production in melanoma cells, and stimulated mushroom tyrosinase activity, which showed its potential utility in preventing hair depigmentation. In addition, DIW showed excellent antibacterial activities against *P. acnes*, *S. aureus*, and *S. epidermidis*, which are acne-causing bacteria. DIW had excellent dose-dependent inhibitory activities of NO production in macrophage RAW cells. These results indicated that DIW may be an attractive acne-mitigating candidate for topical application. Finally, to test the application of DIW as a cosmetic ingredient, HPLC fingerprint and human patch tests were successfully performed in this study with no evidence of toxicity. In conclusion, DIW may be practically applicable as a functional cosmetic ingredient. However, further work is needed to identify the active compound(s) and its mechanisms.

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